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Identification of hepatic molecular mechanisms of action of alpha-tocopherol using global gene expression profile analysis in rats

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Abstract

The recent discovery that vitamin E (VE) regulates gene activity at the transcriptional level indicates that VE may exert part of its biological effects by mechanisms which may be independent of its well-recognised antioxidant function. The objective of this study was the identification of hepatic vitamin E-sensitive genes and examination of the effects of VE on their corresponding biological endpoints. Two groups of male rats were randomly assigned to either a VE-sufficient diet or to a control diet deficient in VE for 290 days. High-density oligonucleotide microarrays comprising over 7000 genes were used to assess the transcriptional response of the liver. Differential gene expression was monitored over a period of 9 months, at four different time-points, and rats were individually profiled. This experimental strategy identified several VE-sensitive genes, which were chronically altered by dietary VE. VE supplementation down-regulated scavenger receptor CD36, coagulation factor IX and 5-α-steroid reductase type 1 mRNA levels while hepatic gamma glutamyl-cysteinyl synthetase was significantly up-regulated. Measurement of the corresponding biological endpoints such as activated partial thromboplastin time, plasma dihydrotestosterone and hepatic glutathione substantiated the gene chip data which indicated that dietary VE plays an important role in a range of metabolic processes within the liver.

Keywords: α-Tocopherol; Rat; Liver; Gene expression; DNA array

1. Introduction

Vitamin E (VE) is considered the most effective lipid-soluble, chain-breaking antioxidant, which protects cell membranes from peroxidative damages [1]. Since its discovery, studies have mainly focused on its antioxidant properties. In 1991 Angelo Azzi's group first described non-antioxidant, cell signalling functions of VE demonstrating that α-tocopherol regulates protein kinase C activity in smooth muscle cells [2]. At the transcriptional level, α-tocopherol modulates the expression of the CD36 scavenger receptor in smooth muscle cells and monocyte-derived macrophages [3], the hepatic α-tocopherol transfer protein (αTTP) [4] as well as the expression of liver collagen alpha-1 [5], collagenase [6] and the α-tropomyosin gene [7]. Recently, a tocopherol-dependent transcription factor (tocopherol associated protein, TAP) has been identified [8].

Methods for the large-scale measurement of gene expression are becoming important tools in the field of free radical research and molecular nutrition [9–12]. In order to obtain a comprehensive understanding of the molecular mechanisms of action of VE, a global gene expression profile in rat liver was obtained using gene chip technology. Various time-point measurements were introduced in the experimental design in order to elucidate whether or not differences in the gene expression profile were manifested consistently over a prolonged period of time. In previous studies, differential changes in gene expression in response to oxidant/antioxi-

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dant treatments were often monitored only at one time-point [13] and in pooled samples [14]. To the best of our knowledge the current study contains a novel design: with animals followed for a period of 9 months at four individual time-points and animals profiled individually at each time-point. Furthermore, changes in gene expression were tested for their ability to cause alterations in the corresponding biological endpoints. Our choice of tissue was guided by the fact that the liver, as the central organ involved in endogenous VE metabolism, is highly susceptible to changes in dietary VE with potential "down-stream" effects on extrahepatic tissues.

2. Materials and methods

2.1. Animal and diets

Two groups of 30 recently weaned male albino rats (Fisher 344, Charles River) with an initial average weight of 50–60 g were randomly assigned to either a vitamin E-containing diet (VE^+) [15] or to a control, vitamin-deficient diet (VE^-) for 290 days. The VE^+ diet contained 60 mg/kg RRR-α-tocopheryl acetate (Vitamin E acetate 950 NS, Roche Vitamins Ltd, Basel, Switzerland) which corresponds to 80 IU/kg diet. The current recommendation for vitamin E by the American Institute of Nutrition is 75 IU/kg in the maintenance diet for growing rats [16]. The VE^- animals received 60 mg/kg of the Vitamin E acetate-free 950 NS vehicle (16% fish gelatine, 8% micro gel E, 1% Sipernat 50) resulting in a final dietary concentration of 1.7 mg/kg RRR-α-tocopheryl acetate. The VE^- and VE^+ rats consumed the diets ad libitum and had free access to water. Rats were maintained under standard conditions at 22 ± 1 °C with 12:12-h dark/light cycles. The Laboratory Animal Care Committees of F. Hoffmann-La Roche Ltd, Basel and the Veterinary Office of Basel-Stadt, Switzerland approved all animal protocols.

Beginning from day 17 of feeding, five animals per group were euthanized every 3 months under isoflurane anaesthesia by withdrawing blood from the vena cava. Livers were removed, rinsed in ice-cold phosphate-buffered saline, pH 7.4 and snap frozen in liquid nitrogen. Samples were stored at −80 °C prior to RNA extraction.

2.2. Vitamin E status in liver and plasma pyruvate kinase activity

Liver tissue was saponified in a methanolic potassium hydroxide solution. The solution was diluted in 35% ethanol and extracted with hexane/toluol. α-Tocopherol was quantified by isocratic HPLC analysis [17] using a Lichrosorb™ Si 60.5 μm, 20 × 4 mm pre-column coupled to a Lichrosorb™ Si 60, 5 μm, 125 × 4 mm column (Stagroma, Reinach, Switzerland) and 3% 1.4-dioxane in *n*-hexane as the mobile phase (flow rate ~ 1.6 ml/min, pressure 35–55 bar). Fluorescence was measured at 330 nm after excitation at 295 nm.

The plasma samples were analysed for α-tocopherol using a routine HPLC method. [18]. Briefly, the plasma proteins were precipitated using ethanol and tocopherol was extracted by liquid/liquid extraction with hexane. After centrifugation, an aliquot of the organic phase was chromatographed isocratically on a normal phase HPLC system. Tocopherol was identified and quantified by fluorimetry (excitation at 298 nm/emission at 326 nm). For quality control, internal control samples were analyzed. The control samples analyzed within each HPLC run indicated that the measured values were within the range of the declared concentrations of the certified samples. Pyruvate kinase (PK) was analyzed in Li-heparinized plasma using a "COBAS-Fara II" centrifugal analyzer as described elsewhere [15].

2.3. Total RNA extraction, cRNA preparation and gene chip hybridisation

Snap frozen tissue samples were homogenized under liquid nitrogen. RNA isolation was performed using phenol–chloroform extraction (TRIzol Reagent, Invitrogen, Basel, Switzerland). Phenol–chloroform-purified RNA was repurified using RNeasy mini spin columns (Qiagen, Basel, Switzerland) and DNase-digested on the columns (RNase-Free DNase Set, Qiagen) according to the manufacturer's description.

A T7-$(T)_{24}$ primer (5′-GGCCAGTGAATTGTAATAC-GACTCACTATAGGGAGG CGG-$(dT)_{24}$-3′) was annealed to 10 μg of total RNA and Superscript II reverse transcriptase (400 U) was utilized to synthesize first-strand cDNA in the presence of DTT, dNTPs and 1 × reaction buffer. Second strand synthesis was performed by adding *E. coli* DNA polymerase I (40 U), *E. coli* ligase (10 U), and RNase H (2 U) in a final reaction containing 1 × second strand buffer in the presence of dNTPs. Finally, strands were blunted using T4 DNA polymerase (10 U) (Superscript™ Microarray Customized Kit, Invitrogen). The resulting cDNA was purified by phenol/chloroform extraction and, subsequently, in vitro transcription was carried out for 3 h using T7 RNA polymerase (Megascript™ T7 Kit, Ambion, Texas, USA), incorporating bio-16-UTPs and bio-11-CTPs (Roche Molecular Biochemicals, Penzberg, Germany). After RNeasy purification, 10 μg of the resulting cRNA was fragmented using 40 mM Tris acetate (pH 8.1), 100 mM potassium acetate and 30 mM magnesium acetate at 95 °C for 35 min. The hybridisation mixture was prepared to contain 100 mM MES buffer, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20, sample cRNA, fragmented bacterial control spikes, the biotinylated oligo 984, herring sperm DNA (0.5 μg/μl; Invitrogen) and acetylated BSA (0.25 μg/μl; Promega) as described in the Affymetrix GeneChip® Expression Analysis Technical Manual. Samples were then hybridised onto Affymetrix GeneChip® Rat Genome U34A arrays for 16 h at 45 °C, with a separate gene chip used for each individual animal. Finally, arrays were washed in the

GeneChip® Fluidics 400 station (Affymetrix) with the EukGE-WS2v3 program and staining was carried out twice with streptavidin R-phycoerythrin (SAPE) using an antibody amplification protocol. Raw fluorescence data were collected by confocal laser scanning (Hewlett Packard, Palo Alto, CA, USA) and analysed with the Affymetrix® Microarray Suite (MAS 4.0).

2.4. GeneChip® microarray expression analysis

Data processing was carried out using the RACE-A analysis tool (Roche Bioinformatics, Basel, CH). The arrays were normalized against the mean of the total sum of Average Difference (AvgDiff) values across all arrays used. Mean average difference values (MeanAvgDiff) were calculated from the replicate samples. Possible outliers were identified using the procedure of Nalimov with a 95% confidence interval. Subsequently change factors (Chgf) for each individual gene were calculated among the different treatment groups using pairwise comparisons and statistical significance was assessed by Student's t test with prior testing for the normal distribution of the data. For a comprehensive analysis, genes with a maximal MeanAvgDiff>20 and differential change fold expression (Chgf) of >0.25; <−0.25 (>25% increase or >25% decrease) combined with a significance level of $P<0.05$ were selected.

Differential expression profile analysis between the two treatment groups was performed at four different time-points (T1 = day 17, T2 = day 91, T3 = day 191, T4 = day 269 of feeding) over the 9-month study period. Data obtained during the different time-points have been compared and only genes consistently up- or down-regulated over a period of time >170 days (3 consecutive time-points) have been taken into consideration for further characterization.

2.5. Plasma testosterone and 5α-dihydrotestosterone levels

Heparinized plasma was collected and stored at −80 °C. Quantification of testosterone and 5α-dihydrotestosterone (5α-DHT) was performed by using a Coat-A Count Total Testosterone (DPC, Los Angeles, USA) and a 5α-DHT ELISA kit (IBL, Hamburg, Germany), respectively, according to the manufacturer's protocol.

2.6. Activated partial thromboplastin time

For measurement of activated partial thromboplastin time (APTT), blood was collected by puncture of the retro orbital vein under light isofluorane anesthesia. The first five free flowing blood drops were discarded. Thereafter, 9 volumes of blood were immediately mixed with 1 volume of 0.13 M sodium citrate and centrifuged at 2000 × g for 30 min. The separated plasma was stored in the refrigerator at +4 °C until analysis. Fifty microliters of plasma was incubated with 50 μl actin FSL reagent (Dade Behring #B4219-2, Marburg, Germany) at 37 °C for 180 s. The reaction was started by adding 50-μl $CaCl_2$-solution (Dade Behring #ORHO 33) and the clotting time recorded using a ball type coagulometer (Amelung KC 4A micro, Lemgo, Germany).

2.7. Plasma triglycerides, total cholesterol and glucose

Heparinized plasma was collected and stored at −80 °C. Quantification of triglycerides, total cholesterol and glucose was performed by using a Hitachi 912 analyser (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocols.

2.8. Glutathione analysis

Glutathione (GSH) and glutathione disulfide (GSSG) in liver homogenates were measured by HPLC with UV detection [19]. Liver tissue was homogeneized in ice-cold PBS. One hundred microliters of liver homogenate was added to an equal volume of trichloroacetic acid 5% containing 0.1 mM desferal and centrifuged at 4000 × g for 10 min at 4 °C. Fifty milligrams of $NaHCO_3$ and 50 μl freshly prepared iodo-acetic acid (108 mM) were added to the sample, vortexed and incubated in the dark for 1 h. After adding 200 μl of Sanger reactant (2,4-dinitro-1-fluorobenzene in 0.375% ethanol), samples were vortexed and let for reaction over night in the dark at room temperature. After reaction samples were centrifuged at 2000 × g for 10 min at 4 °C, the supernatant was collected, filtered and injected in the HPLC system using the following conditions. Injection volume: 100 μl, flow: 1.2 ml/min, solvent A: 25% methanol in water and solvent B: 2 M sodium acetate/methanol (36:64, pH 4.6), the column was a Waters Spherisorb NH_2, 5 μm (15 × 0.46 cm), the elution gradient: from 75% A to 95% B in 30 min, and the detection wave length was 365 nm.

2.9. Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.). The data were checked for normality and log-transformed where appropriate. Statistical analysis was carried out using ANOVA and Student t tests (SPSS for Windows version 10.0). Differences were considered significant if $P<0.05$.

3. Results

Feed intake was not significantly different between the two experimental groups. Furthermore, body weight gain was similar between VE^- and VE^+ animals within the 9-month experimental trial as shown in Table 1. Rats fed the VE^+ diet exhibited significantly higher plasma VE levels and significantly lower plasma pyruvate kinase activities as compared to controls receiving the VE^- diet. Differences in

Table 1
Effect of dietary vitamin E on body weight gain, plasma vitamin E and plasma pyruvate kinase activity as well as liver vitamin E concentrations in rats at various time points (T1–T4)

	Body weight (g)		Plasma vitamin E (μmol/l)		Plasma pyruvate kinase (U/ml)		Liver vitamin E (nmol/g tissue)	
	VE^-	VE^+	VE^-	VE^+	VE^-	VE^+	VE^-	VE^+
T1 (Day 17)	124.8 ± 4.60	124.2 ± 3.93	3.20 ± 0.19	18.1* ± 1.52	2.36 ± 0.05	2.28 ± 0.11	2.08 ± 2.24	44.95* ± 14.95
T2 (Day 91)	321.7 ± 2.32	325.2 ± 6.70	0.20 ± 0.04	31.6* ± 1.34	24.4 ± 0.84	1.80* ± 0.08	1.75 ± 1.53	71.52* ± 13.45
T3 (Day 191)	354.7 ± 15.2	352.5 ± 18.91	0.53 ± 0.09	21.6* ± 1.92	12.4 ± 2.28	1.47* ± 0.09	1.34 ± 0.50	82.93* ± 14.8
T4 (Day 269)	387.2 ± 8.94	388.4 ± 11.85	0.20 ± 0.09	27.4* ± 3.17	18.7 ± 0.80	1.90* ± 0.26	0.35 ± 0.20	83.91* ± 15.86

Values are group means ± S.E., $n = 5$. At T0 average BW: 60 ± 4 g, plasma VE and plasma PK as well as liver VE were not determined.
* Indicates significant differences of $P < 0.05$ between the two treatment groups.

dietary VE supply were also reflected by significant differences in hepatic VE concentrations. Liver VE levels increased time-dependently in the VE^+ group.

Out of the ~ 7000 genes represented on the GeneChip®, only 14 annotations comprising five genes (1 × gamma-glutamylcysteinyl synthetase, 1 × GSH synthetase, 1 × fac-

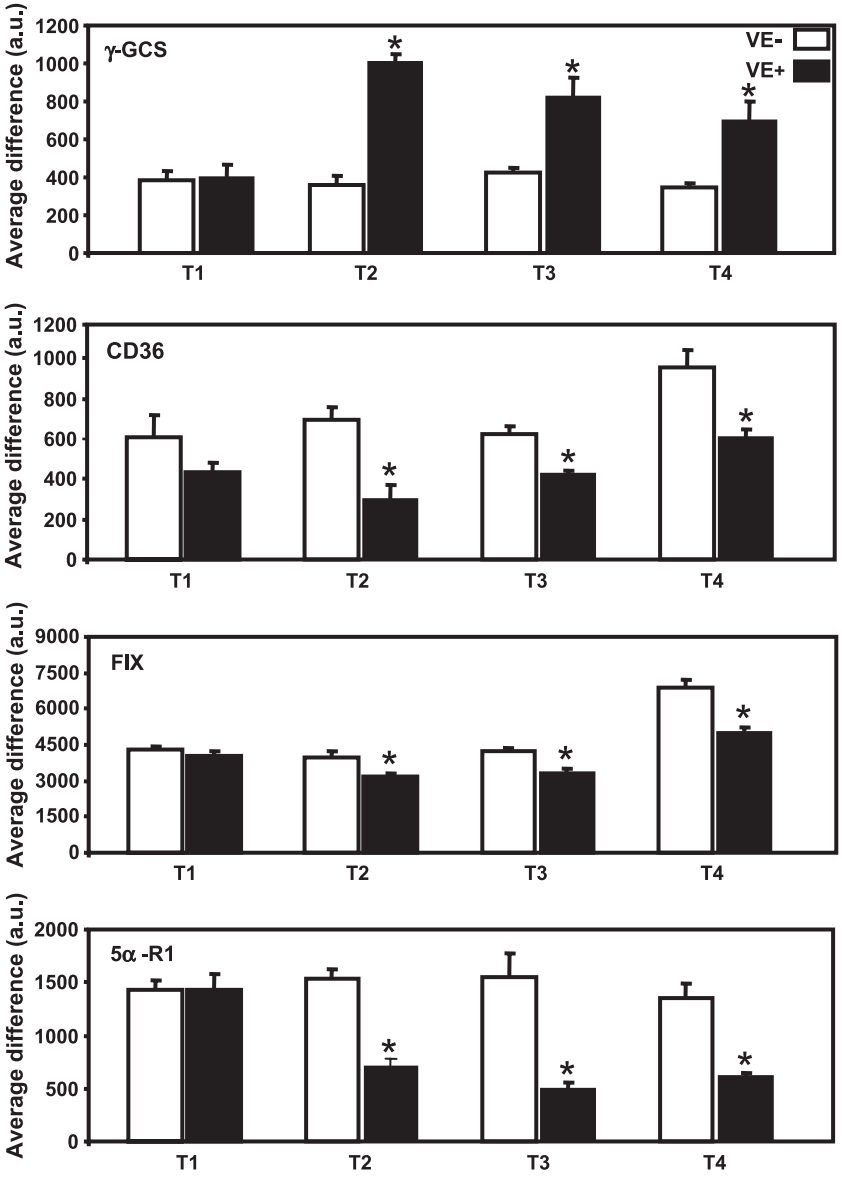

Fig. 1. Effect of dietary vitamin E on hepatic regulatory subunit gamma-glutamylcysteinyl synthetase (γ-GCS), scavenger receptor CD36, coagulation factor IX (FIX) and 5-α-steroid reductase type 1 (5α-R1) mRNA levels in rat liver measured at four different time-points. Values are group means ± S.E., $n = 5$; *Significant differences ($P < 0.05$) between VE^- and VE^+ rats within one time point. T1 = day 17, T2 = day 91, T3 = day 191 and T4 = day 269 of feeding.

tor IX, 1 × 5-α-steroid reductase type 1, 3 × scavenger receptor CD36) and seven expressed sequence taqs were consistently differentially expressed in response to VE in at least three consecutive time-points. The regulatory subunit of gamma-glutamylcysteinyl synthetase (γ-GCS), scavenger receptor CD36, 5-α-steroid reductase type 1 (5α-R1) and coagulation factor IX (FIX) were followed up in more detail. We have selected these four genes since their gene products are well characterized and metabolic endpoints are available in order to confirm whether differences in mRNA levels were associated with any 'down-stream' functional changes.

The time course of the gene expression profiles of γ-GCS, CD36, FIX and 5α-R1, over the 9-month experimental period is given in Fig. 1. At T1 there were no significant differences between VE^+ and VE^- rats for any of these four genes. However, between T2 and T4 a consistent up-regulation (γ-GCS) and down-regulation (CD36, FIX and 5α-R1), respectively, was evident in VE^+ animals as compared to controls.

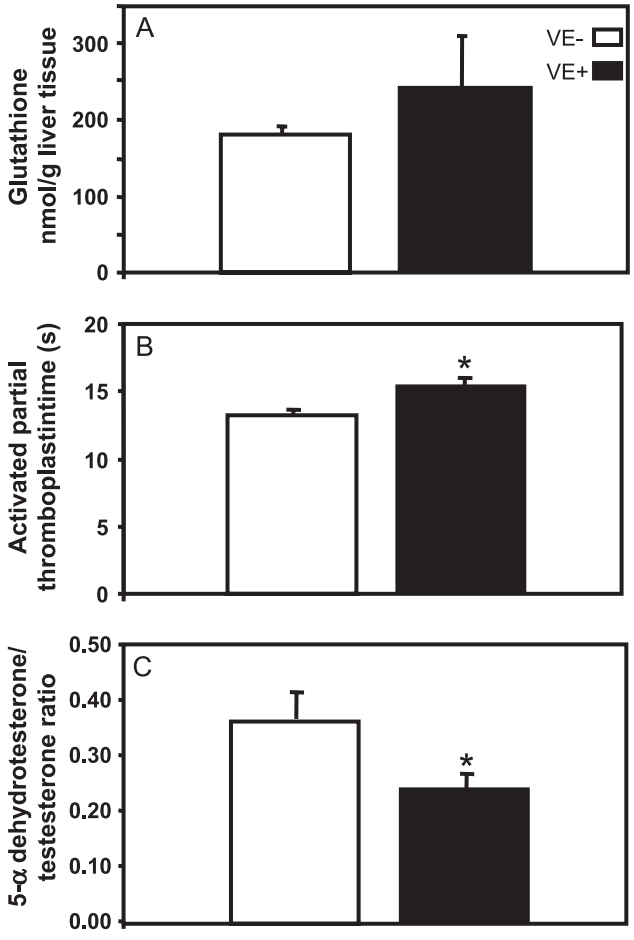

Fig. 2. Effect of dietary vitamin E on liver Glutathione levels (A), partial thromboplastin time (B) and plasma normalised 5α-dihydrotestosterone (5α-DHT/testosterone ratio) (C) in rats. Values are group means ± S.E., $n = 5$; *Significant differences ($P < 0.05$) between VE^- and VE^+ rats at T4.

Subsequently, differences in the gene expression levels observed by GeneChip® technology were confirmed by real-time PCR (data not shown) and substantiated by functional parameters. VE^+ rats exhibited 20% higher hepatic GSH levels as compared to VE^- rats (Fig. 2A), whereas liver GSSG levels ($VE^- = 25.1 \pm 7.1$ nmol/g vs. $VE^+ = 28.2 \pm 3.1$ nmol/g) remained largely unchanged. As summarized in Fig. 2B, a significant decrease in FIX mRNA levels in VE^+ rats was accompanied by a significant increase in activated partial thromboplastin time APTT. Furthermore, rats receiving the VE^+ diets showed a significant decrease in plasma normalised 5-α-dihydrotestosterone (5α-DHT/testosterone ratio) as compared to VE^- animals (Fig. 2C). Since the biological function of liver CD36 has not yet been fully established, we based our endpoint analyses on findings made in CD36-deficient mice [20,21] and humans with non-functional CD36, where plasma lipids, triglycerides, free fatty acids and glucose levels were altered [22]. However, under the conditions investigated, changes in CD36 gene expression were not accompanied by any significant differences in plasma triglycerides, total cholesterol and fasting glucose (data not shown).

4. Discussion

Our experimental design monitoring differential gene expression at four individual time-points over a period of 9 months with each rat profiled individually allowed chronic changes in mRNA levels in response to dietary VE to be monitored. It is of interest to note that under in vivo conditions, changes in differential gene expression are rather small and often below 2 [23,24], a threshold factor often used in cell culture experiments. Interestingly after 17 days (T1) the mRNA levels of γ-GCS, CD36, 5α-R1 and FIX were similar between VE^- and VE^+ animals. However, these transcripts were consistently altered between T2 (day 91) and T4 (day 269). Fischer et al. [13] reported that dietary VE deficiency over a 7-week period in rats did not result in any significant changes in the gene expression profile among 465 liver genes monitored with a DNA micro-array. On the basis of these and the current data, it seems likely that only long-term changes in VE exposure result in substantial and consistent changes in gene expression. Of particular interest is the variety of biological pathways which seem to be influenced by VE. The potential links between VE and androgen metabolism, blood coagulation and redox homeostasis are already known although the molecular mechanisms have not been elucidated. The present study contributes to the current understanding of the mechanisms of action behind these observations.

Plasma levels of vitamin E were used to evaluate the adequacy of the supplementation in the present study. With an average plasma VE concentration of 26 μmol/l over the last three time points (T2–T4), the VE levels measured in

the current experiment match the ~ 25 μmol/l values found in healthy humans not supplemented with VE [25].

Pyruvate kinase activity is an indicator for vitamin E deficiency. However, the correlation between PK activity and degree of VE deficiency is a matter of debate. PK is released by damaged erythrocytes as well as myocytes and many more cell types. The observed differences in PK activity at day 17 (VE^- group) is essentially caused by an increased hemolysis of red blood cells. Hemolysis is known to be an early indicator of mild VE deficiency [26]. With ongoing, more profound VE depletion, PK is also released by damaged myocytes (time T2–T4). The classical signs of severe VE deficiency such as Ataxia and body weight loss were not observed during the 290 days of differential VE supplementation.

4.1. Gamma-glutamyl cysteinyl synthetase

De novo synthesis of GSH from its constitutive amino acids glutamate, cysteine, and glycine involves two sequential reactions. The first is catalysed by γ-GCS and is rate-limiting, and the second by GSH synthetase [27]. VE supplementation resulted in a significant up-regulation of the expression of γ-GCS as well as GSH synthetase (data not shown). It has been previously demonstrated that dietary VE increased GSH levels in rat liver [28]. Thus, the augmentation of cellular GSH by VE may offer an important dietary strategy to enhance the ability of the liver to cope with oxidative stress. The importance of intracellular GSH in preventing apoptosis [29] and inflammation [30] has been appreciated for a long time and both the dietary and pharmacological manipulation of GSH synthesis have received much attention. Increased levels of GSH in the liver have been associated with increased tolerance to oxidative stress and to xenobiotics [31]. In vitro experiments have demonstrated a synergism between GSH and VE, indicating that GSH can recycle VE radicals [32,33] via ascorbic acid. The current findings suggest an alternative pathway and a novel molecular mechanism of VE/GSH interactions within the so-called cellular antioxidant network [34,35], i.e. that VE up-regulates GSH synthetase and γ-GCS. It has been reported that γ-GCS mRNA levels decline with age in the liver [36] and that dialysis patients who are exposed to high oxidative stress show low concentration of GSH [37]. Further studies investigating whether dietary VE supplementation can delay or prevent the age-related or oxidation-related decline in γ-GCS are therefore merited.

4.2. Scavenger receptor CD36

Alpha-tocopherol exposure in human monocyte cell lines and primary monocyte derived macrophages has been shown to decrease CD36 expression and decrease the extent of modified low density lipoprotein (LDL) accumulation. [38]. In an in vitro study using aortic smooth muscle cells, a positive effect of α-tocopherol in reducing CD36 expression was also evident [3]. Lack of effect of β- or γ-tocopherol, which have comparable antioxidant potential, indicates that the α-tocopherol acts via a non-oxidant-dependent cell signalling pathway as first described by Boscoboinik et al. [2].

In the current study VE supplementation resulted in a consistent down-regulation in CD36 expression with a significant 35–70% decrease relative to control animals between T2 and T4. Although CD36 is thought to be present in most tissues, embedded in the cholesterol rich domains of membranes [38], little knowledge exists on the function of CD36 in liver cell metabolism. In our model, comparison of plasma triglycerides, total cholesterol and glucose levels in VE^+ animals versus controls did not result in any significant changes. This result is in line with observations made in other studies where long-term VE supplementation did not result in significant changes in blood lipids concentration [25]. Alterations in blood lipids have been observed in CD36 null animals and in animals overexpressing the CD36 gene. However, it is likely that the approximate 50% alteration in CD36 expression in the current study would not impact total circulating lipid levels, given that the LDL-receptor family rather than CD36 are primarily responsible for the receptor-mediated clearance of lipoproteins from the circulation.

The precise molecular mechanism by which α-tocopherol impacts on CD36 expression is uncertain although an effect on either PKC or PPAR-γ is likely [39]. Alternatively, this effect may be the result of reduced circulating oxidized LDL in the VE^+ animals compared to the VE^- animals. Alpha tocopherol is known to be the main antioxidant component of LDL, therefore, decreased CD36 expression in the VE supplemented group may simply reflect lower circulating oxidized LDL levels.

The consequence of the CD36 changes in the liver is currently unknown but it may be of greater functional consequence in other cell types. If the down-regulation of hepatic CD36 expression observed in the current study was reflected in other tissues such as the monocyte-macrophage, it could lead to a decreased accumulation of LDL in the arterial intima with a potential benefit for the development of atherosclerosis.

4.3. Coagulation factor IX

Coagulation factor IX (FIX) was found to be down-regulated in VE^+ animals. FIX is a vitamin K-dependent blood clotting protein, which plays an essential role in the activity of the intrinsic coagulation pathway. Congenital deficiency of FIX, haemophilia B, is characterized by the pronounced tendency of bleeding [40]. On the other hand, high levels of plasma FIX have been associated with an increased risk of venous thromboembolism [40]. It is well known that VE possesses anticoagulant properties and a number of mechanisms have been proposed such as inhibi-

tion of platelet adhesion and aggregation [35]. In addition, VE is thought to act as an antagonist of the vitamin K-dependent gamma-carboxylase, which is required to activate enzymes of the blood clotting cascade including FIX [40]. In this regard, it has been suggested that the inhibitory activity comes from an oxidized form of VE, α-tocopherolquinone, which may be present in trace amounts in VE preparations or formed within the body during VE metabolism [41]. In the current study, VE resulted in a moderate and long-lasting repression of the FIX transcript. In order to test the relevance of the down-regulation of FIX in response to VE, activated partial thromboplastin time (APTT) was assessed, which is a well-accepted screening test for FIX deficiency. Rats supplemented with VE showed a ~1.2-fold increase in APTT. Inhibition of APTT is of recognised value in the prevention and treatment of thromboembolic events [42]. Overall, our present data support, on a molecular level, the concept that VE may posses some anticoagulant activity. Anticoagulant properties of VE seem to be partly mediated by a down-regulation of FIX.

4.4. 5-α-Steroid reductase type 1

5-α-Steroid reductase type 1 (5α-R type1) catalyses the irreversible conversion of testosterone to 5α-dihydrotestosterone (5α-DHT), the most potent androgen known. Two isoenzymes have been identified, namely 5α-R type 1 (5α-R1) and 5α-R type 2 (5α-R2), which catalyse the same reaction, but possess distinctive biochemical properties and different tissue distributions [43]. 5α-R1 is widely distributed and found in many tissues such as hair follicles, sebaceous glands and the liver while 5α-R2 is primarily located in the male reproductive tract such as the prostate, seminal vesicle and epididymis [44]. Overproduction of 5α-DHT has been implicated in the progression of benign prostatic hyperplasia (BPH), prostate cancer, acne, hair loss, hirsutismus and breast cancer [45–47]. In humans, both isoenzymes are expressed in the liver while in rats only 5α-R1 is detected [48]. In the liver, 5α-Rs are believed to exert a double physiological function, (i) the conversion of testosterone to 5α-DHT with local and systemic activity and (ii) the inactivation of excessive blood steroid levels by the consecutive action of 5α-Rs and 3α-hydroxysteroid dehydrogenase thereby yielding weaker androgens [49].

In the present study dietary VE was associated with a consistent transcriptional decrease in 5α-R1 mRNA levels. In order to test the physiological relevance of this alteration at the enzyme activity level, we have quantified plasma testosterone and 5α-DHT in VE^+ versus VE^- animals. It has already been shown that VE supplementation significantly decreased plasma levels of testosterone per se in humans, however, the underlying mechanism of action remains unknown. Since most of 5α-DHT is derived from the conversion of testosterone, a decrease in 5α-DHT plasma concentrations may simply reflect a decrease in the precursor hormone rather than the inhibition of the enzymatic conversion. In order to test for this possibility, plasma 5α-DHT levels were normalised by plasma testosterone levels, i.e. 5α-DHT/testosterone, in each animal individually. Importantly, the normalised 5α-DHT level was significantly decreased in VE^+ animals supporting the hypothesis of a decrease in 5α-Rs activity due to VE. Epidemiological and intervention studies have shown a potential positive effect of VE supplementation in the prevention of prostate cancer [46] and in the relief of BPH symptoms [50]. Several mechanisms of action have been postulated such as protection of DNA damage by free radicals, induction of apoptosis, modulation of immune function and interference with hormone production [51]. The latter hypothesis was supported by the findings of the ATBC intervention study where men who received α-tocopherol had significantly lower serum androstenedione and testosterone levels than did those who received a placebo [52]. These findings have been proposed as an explanation for the reduction in prostate cancer observed in this study. The current data suggest that dietary VE acts as a suppressor of 5α-R1 isoenzyme at the transcriptional level possibly affecting the conversion of testosterone to 5α-DHT.

4.5. α-TTP and collagen-alpha1

Several genes including α-TTP, collagen-alpha1, alpha tropomyosin as well collagenase have been reported in the literature to be vitamin E-sensitve. However, these genes were not significantly altered in their expression in the present rat experiment in response to differences in dietary vitamin E supply.

We found a strong hybridization signal for α-TTP in both VE^- and VE^+ livers, however, no significant difference between the two groups was observed. Our results confirm those of Shaw and Huang [4]. In animals fed either a VE-deficient or a VE-adequate diet (100 mg/kg feed VE) for 5 weeks, no difference in α-TTP mRNA expression was found. Modulation of liver α-TTP expression was only observed when VE-deficient animals where re-supplemented with VE [4]. However, in our study, we did not re-supplement VE deficient animals.

Chojkier et al. [5] did compare the mRNA levels of collagen-alpha1 between livers of rats fed either with a VE-deficient diet or a VE-supplemented diet (>5000 mg/kg feed VE) for 8 weeks. They found a significant, 70% decrease, in collagen-alpha1 expression in VE supplemented animals. In the present study a moderate and consistent decrease in the expression of the collagen-alpha1 gene between T2 and T4 was observed in the liver of VE^+ animals. Collagen-alpha1 showed an average decrease of ~30% ($P<0.05$) at time-points T2 and T4. Since statistical significance was not found at T3, this gene was not considered for further analysis. A possible explanation for the differences in the down-regulation of collagen-alpha1 in response to vitamin E between our experiment and the results by Chojkier et al. [5] might be related to differences in the dietary vitamin E concentration (60 vs. 5000 mg/kg diet).

4.6. Alpha-tropomyosin and collagenase

Azzi's group was the first to show that VE is able to modulate the expression of alpha-tropomyosin in rat vascular smooth muscle cell line. The increase in mRNA expression was shown to be transient and limited within the first hours of VE stimulation [7]. Tropomyosin is a muscle-specific gene and is not expected to be of physiological relevance in liver. Our mRNA expression data confirm that tropomyosin is expressed only in trace amount in rat liver. In addition, short-term, transient changes in gene expression cannot be detected by our experimental design. Azzi's group has demonstrated that VE down-regulates collagenase expression in human fibroblast cell culture [6]. Also in this case, the biological relevance of this gene in the liver, if any, is unknown. In our study its expression level was at the limit of detection.

5. Conclusion

Advances in expression microarray technology have allowed us to investigate genes differentially expressed in rat liver in response to VE, thereby offering the possibility of more insight into the biological functions of alpha-tocopherol. Dietary VE leads to a significant up-regulation of γ-GCS, the rate-limiting enzyme in GSH synthesis. Furthermore, VE induced a down-regulation of coagulation factor IX and 5-α-steroid reductase type 1 mRNA levels. These VE-related changes in gene expression were accompanied by significant alterations in liver GSH, plasma activated partial thromboplastin time and normalised 5α-DHT levels, respectively. Overall, the current study reveals that dietary VE has important long-term effects on liver gene expression with potential downstream effects on extra-hepatic tissues and illustrates the application of genome-wide expression profiling when investigating the biological action of essential dietary micronutrients.

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